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Radioenzymatic Determination of Epinephrine, Norepinephrine and Dopamine in 0.1 ml Plasma Samples
Plasma Catecholamine Response to Submaximal and Near Maximal Exercise

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Summary: A sensitive enzymatic isotope derivative method for simultaneous determination of epinephrine, norepinephrine, and dopamine in 0.1 ml plasma samples is described. The assay consists basically of

1. conversion of epinephrine, norepinephrine, and dopamine into their respective methyl-derivates in the presence of catechol-O-methyltransferase and S-adenosylmethionine-[^{3}H]methyl,

2. extraction of the methylated ^{3}H labelled amines with diethyl ether,

3. separation by thin-layer chromatography,

4. measurement in a beta radiation scintillation counter.

The effect of standing upright and of submaximal and near-maximal steady state exercise on plasma epinephrine, norepinephrine, dopamine and plasma renin was studied in 16 young adults aged 22 to 34 years and 9 physically well trained boys 16–17 years old. Standing upright for 2 min did not result in significant changes in either epinephrine, norepinephrine, dopamine or plasma renin activity compared with supine (basal) values. During exercise, epinephrine, norepinephrine, dopamine and plasma renin activity increased exponentially with the work load, peak values being attained at the highest load or immediately after cessation of exercise. The respective maximal values for plasma renin activity and epinephrine and norepinephrine levels were approximately 3, 6 and 10 fold higher than basal values in the adults, and rose as high as 4, 10 and 20 fold in the boys. Peak dopamine values were only 2–3 times higher than the basal values in both adults and boys.

It is concluded that the method described provides a suitable tool for a quantitative assessment of adreno-sympathetic tone during physiological and pathophysiological conditions.

Radioenzymatische Bestimmung von Adrenalin, Noradrenalin und Dopamin in 0,1 ml Plasmaproben
Plasma-Katecholaminkonzentrationen und Plasma-Renin-Aktivität in Ruhe
und bei submaximaler und nahezu maximaler Belastung

Zusammenfassung: Eine empfindliche radioenzymatische Methode zur gleichzeitigen Bestimmung von Adrenalin, Noradrenalin und Dopamin in Plasmamengen von bis zu 0,1 ml wird beschrieben. Die grundsätzlichen Schritte sind:

1. Umwandlung von Adrenalin, Noradrenalin und Dopamin in die jeweiligen Methylderivate unter Einwirkung von Catechol-O-methyl-transferase und S-adenosyl-methionin-[^{3}H]methyl,

2. Extraktion der methylierten und ^{3}H-markierten Amine mit Hilfe von Diethylether,

3. ihre Trennung durch Dünnschichtchromatographie,

4. Konzentrationsmessung in einem Beta-Strahlen-Scintillationszähler.

Die Änderungen von Adrenalin, Noradrenalin und Dopamin im Plasma und zusätzlich der Renin-Aktivität im Plasma im Vergleich zu den Basalwerten im Liegen wurden nach 2minütigem Stehen und während submaximaler und nahezu maximaler „steady state" Belastung im Sitzen am Fahrradergometer bei 16 Erwachsenen im Alter von 22–34 Jahren und bei 9 gut trainierten Jungen im Alter von 16–17 Jahren in Beziehung zur Veränderung von Herzfrequenz und arteriellen Drucken untersucht. Während ein 2minütiges Stehen keine signifikanten Änderungen von Adrenalin, Noradrenalin, Dopamin oder der Renin-Aktivität im Plasma ergab, kam es bei Belastung am Fahrradergometer zu einem exponentiellen Anstieg der Katecholamine und der Renin-Aktivität im Plasma, wobei die Maximalwerte bei der höch-

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sten Belastungsstufe oder unmittelbar nach Abschluß der Arbeit erreicht wurden. Renin-Aktivität im Plasma, Adrenalin und Noradrenalin stiegen bei den Erwachsenen um das 3, 6 und 10fache und bei den Jungen sogar um das 4, 10 und 20fache der Ausgangswerte. Dagegen waren die Höchstwerte für Dopamin nur etwa 2–3mal höher als die Ausgangswerte und in den beiden Gruppen nahezu gleich.

Die beschriebene Methode ermöglicht die quantitative Beurteilung der Aktivität des sympathischen Systems und seiner Veränderungen unter physiologischen und pathophysiologischen Bedingungen.

Introduction

The recent development of enzymatic isotope derivative methods (1) has resulted in a significant improvement in both the sensitivity and accuracy of catecholamine assay, as compared with fluorimetric methods; it has thus provided a sensitive tool for the measurement of epinephrine and norepinephrine in plasma and tissues and hence for the study of the role of adrenergic stimulation under physiological and pathophysiological conditions. Some of these techniques are based on the conversion of epinephrine and norepinephrine by the enzyme phenylethanolamine-N-methyltransferase (2). Others involve the 3-O-methylation of epinephrine, norepinephrine and dopamine by the enzyme catechol-O-methyltransferase (3, 4, 5). In both cases the enzymatic reaction is carried out in the presence of labelled S-adenosylmethionine which acts as a methyl donor.

This report describes a radioenzymatic method for the simultaneous determination of epinephrine, norepinephrine and dopamine based on the 3-O-methylation of the amines by catechol-O-methyltransferase. By using diethyl ether as the organic solvent for extraction and other modifications, the sensitivity of the assay was improved compared with that originally described (3, 4). It allows the determination of 0.1 nmol/l of epinephrine, norepinephrine and dopamine; the plasma volume required has been reduced to as little as 0.1 ml. Also, by reducing the number of analytical steps, the method is simpler than that of *Da Prada & Zürcher* (5).

Furthermore, this paper reports the application of the method for the study of the adrenergic response to submaximal and near maximal exercise in adolescents and young adults.

Materials and Methods

Catecholamine assay

Materials

Aquasol 2 and S-adenosyl-methionine-[^{3}H]methyl (specific activity: 277.5–425.5 TBq/mol = 7.5–11.5 Ci/mmol): New England Nuclear Corporation; 3-methoxy-tyramine chloride, dithiothreitol and pargyline: Sigma; metanephrine and nor-metanephrine: Calbiochem; epinephrine-bitartrate, *L*-norepinephrine-*L*-tartrate, 3-hydroxy-tyramine chloride (dopamine) and Silica gel GF plates for thin-layer chromatography: Merck-Schuchart; Bio Rad columns for urine catecholamine assay: Kemila.

All other chemicals were of analytical grade; all reagents were prepared from glass distilled water.

Catechol-O-methyltransferase was isolated from rat liver according to the ammonium sulfate fractionation method of *Axelrod & Tomshick* (6). The 30–35 per cent precipitate was redissolved in distilled water and subsequently dialysed against potassium phosphate buffer (pH 7.0) containing 0.1 mmol dithiothreitol; the final product was adjusted to pH 8.1 using 2.0 mol/l Tris/HCl buffer of pH 8.2, centrifuged and stored in 2 ml aliquots at − 20 °C.

Handling of blood samples

Blood samples were immediately transferred into chilled tubes containing reduced glutathione and heparin prepared in advance by freeze drying. The amount of glutathione was adjusted to give a final blood concentration of 5 mmol/l. The tubes were immediately placed in an ice bath, the solution of glutathione and heparine being facilitated by intermittent inversion and gentle shaking. Plasma was separated by centrifugation at 4 °C, transferred to plastic tubes and frozen. Prior to analysis the samples were thawed in an ice bath at 4 °C, centrifuged and immediately assayed. No significant changes in catecholamines were detected when the separation of plasma was performed within 60 minutes, provided the tubes were stored at 0–4 °C. Plasma can be stored frozen for months without any measurable effect on the catecholamine content.

Analysis

Basically, the analysis comprises

1. methylation of epinephrine, norepinephrine and dopamine,
2. extraction of the methylated catecholamines with diethyl ether,
3. separation by thin layer chromatography and
4. measurement in a scintillation counter.

Methylation was performed in disposable glass tubes (10 × 80 mm) containing a lyophilized medium of 55 µmol of pargyline, 250 µmol of glutathione 6 µmol of $MgCl_2$, 1 µmol of dithiothreitol, 10 µl of 2 mol/l pH 8.2 Tris/HCl buffer and approximately 50 µl of catechol-O-methyltransferase, the volume of the latter varying according to the enzyme activity obtained.

100 µl of plasma and finally 5 µl (55.5 kBq = 1.5 µCi) of S-adenosylmethionine-[^{3}H]methyl were added to the preloaded and chilled tubes and incubated in a shaking waterbath at 37 °C for 90 min.

Subsequently 100 µl of a reference (carrier) solution containing 0.8 µmol each of metanephrine, nor-metanephrine and 3-methoxy-tyramine, and 25 µl of glacial acetic acid were added; the tubes were placed into a bath of boiling water for 5 min and the pH raised to approximately 10 by means of 500 µl of 0.6 mol/l pH 12 borate buffer and 80 µl of 5 mol/l NaOH. After mixing and centrifugation, 600 µl-aliquots of the supernatant were transferred to disposable glass tubes containing 1 g of K_2HPO_4.

Extraction was performed by adding 6 ml of peroxide-free diethyl ether and shaking the capped tubes vigorously in a horizontal shaker for 15 minutes. After centrifugation 5 ml of the ether phase were transferred to another set of glass tubes and taken to dryness at approximately 50 °C under a stream of air.

Separation and measurement: the residues were dissolved in 200 ml of 95 ethanol (950 ml/l), applied to the chromatographic plates and bidimensionally developed in a bath of isopropanol, *n*-butanol, water and formic acid (60 ml + 20 ml +

19 ml + 1 ml) and subsequently in a bath of *n*-butanol, ethyl acetate and ammonium hydroxide (60 ml + 20 ml + 20 ml) for 7 and 3 hours respectively. From 13 consecutive separation procedures the R_F-values in the first dimension were 0.59 ± 0.02 (mean and standard deviation) for both nor-metanephrine and 3-methoxy-tyramine, and 0.48 ± 0.02 for metanephrine; in the second dimension, 0.25 ± 0.01 for nor-metanephrine and metanephrine, and 0.33 ± 0.01 for 3-methoxytyramine.

After drying, the areas corresponding to metanephrine, normetanephrine and 3-methoxy-tyramine were localized under UV-light. The spots were finally scraped into counting vials and mixed with 1 ml of ethanol (950 ml/l) to elute the metanephrines from the silica gel. After approximately 1 hour 10 ml of Aquasol 2 were added, the vials shaken and radioactivity (^3H) measured in a scintillation counter (Intertechnique SL 30).

Standards, calculations, quality criteria

In addition to the assay samples, a blank (pooled) plasma, a standard (pooled) plasma (external standard), a standard plasma of the same individual (internal standard) and a previously assayed (known) control plasma were routinely analyzed. Epinephrine, norepinephrine and dopamine were selectively removed from blank- and standard plasmas by passing them through two Bio-Rad columns. Standards were prepared by adding 5 ml of 0.01 mol/l HCl containing 5 moles of each epinephrine, norepinephrine and dopamine to the tubes prior to incubation.

The radioactivity determined in both samples and standards was corrected for the activity found in the corresponding blank. The concentration of amines in each sample was then calculated, using the radioactivity of the internal standard. The radioactivity encountered in the blank and internal standard plasma was in the order of 80–120 and 12 000–15 000 counts/min respectively.

The linearity was regularly tested by adding known mixtures of catecholamines to plasma in the range of 0.1 to 10 pmol. A typical result is shown in figure 1. The slope coefficient (counts · min^{-1} · nmol · l^{-1} plasma) is in the order of 140 for norepinephrine and 150 for epinephrine and dopamine. However, the slope of the standard curves varies slighthly from day to day which might be due to slight variations in the recovery of the methylated catecholamines. Determined from 10 consecutive experiments the recovery was 65 ± 12, 68 ± 12 and 58 ± 10% for nor-metanephrine, metanephrine and 3-methoxy-tyramine respectively. The detection limit of norepinephrine, epinephrine and dopamine in plasma is in the order of 0.1 nmol/l.

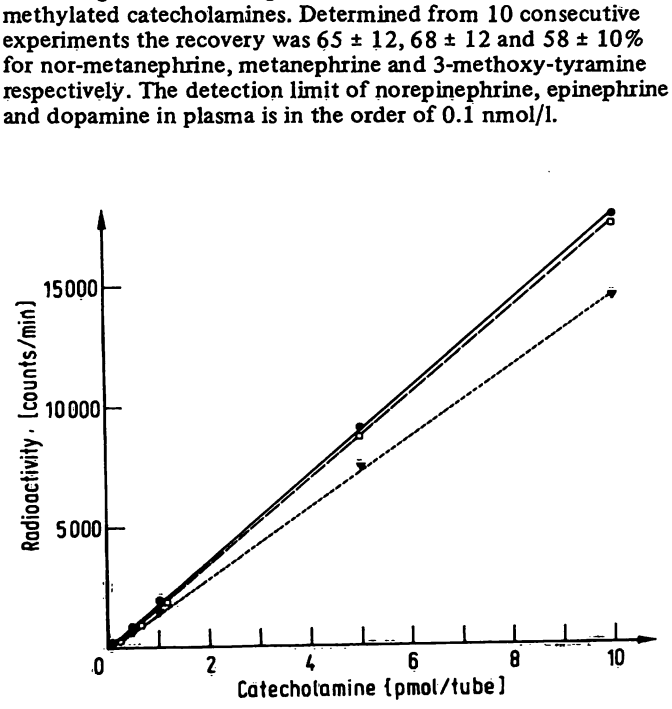


Fig. 1. Linearity of the assay for epinephrine (•—•), norepinephrine (▲------▲) and dopamine (□—□) tested in a standard reaction mixture. Each value represents the mean of a triple determination.

The coefficient of variation calculated from 15 determinations on 0.1 ml plasma samples obtained during resting conditions was 18.6% for epinephrine and 9.8% for norepinephrine at mean concentrations of 0.58 and 2.2 nmol/l respectively, and 5.2% for epinephrine and 5.0% for norepinephrine at mean concentrations of 7.5 and 26.4 nmol/l respectively (plasma sampled after exercise stimulation). For dopamine the cofficient of variation was of the same order of magnitude as for epinephrine.

Subjects and experimental procedure

Plasma epinephrine, norepinephrine, dopamine and plasma renin activity were measured in 2 different groups of subjects:

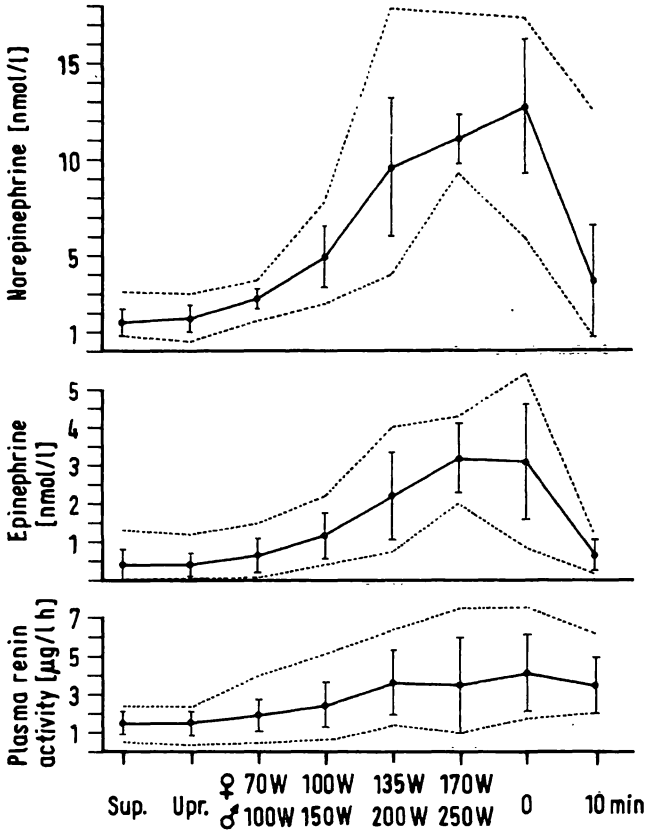
(1) 16 healthy volunteers, 8 men and 8 women, aged 22 to 34 (mean 28) years having normal physical activity,

(2) 9 physically well-trained (maximal oxygen uptake, $\dot{V}_{O_2}$, = 59 ml/kg body weight) boys aged 15 to 16 years, under different conditions: at rest after 10 minutes lying in the supine position, after 2 minutes standing in the erect posture, as well as during and after steady state exercise in the sitting position on a bicycle ergometer. Exercise was performed with at least 4 but mostly 5 increasing work loads, the highest one corresponding to about 85–90 per cent of the individual's maximal working capacity in the adults, and to 95–100 per cent in the group of adolescents. Exercise lasted for 6 minutes at each load; thus the total period of exercise exceeded 24 minutes in every case. Work loads were 35, 70, 100, 135, 170 W and 50, 100, 150, 200, 250 W for the females, and the men and boys, respectively. Heart rates were obtained from continuous ECG recordings, blood pressures were measured with the *Riva-Rocci* cuff method, blood samples were drawn from a catheter inserted percutaneously in an anticubital vein and processed as described. The plasma renin activity was determined by radioimmuno-assay (7).

Results

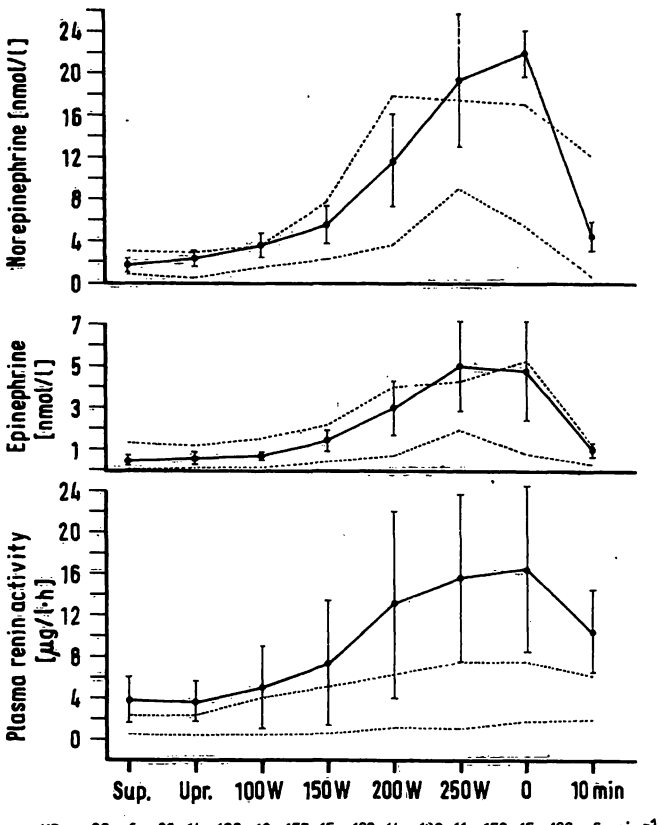
Plasma epinephrine, norepinephrine and renin activity with corresponding heart rates and arterial blood pressures as measured during resting conditions and during and following nearmaximal exercise in the adults and boys are displayed in figure 2 and figure 3 respectively. Table 1 shows the corresponding plasma dopamine data.

There was no significant change in plasma renin activity, epinephrine or norepinephrine after standing for 2 min in the upright position, either in the adults or the boys, although in the adult group, the heart rate increased significantly from 67 ± 8 to 84 ± 16. During exercise, plasma norepinephrine, epinephrine and renin activity increased almost exponentially with the work load, peak values always being attained at the highest load or immediately after cessation of exercise. 10 minutes after work plasma norepinephrine and renin activity were still significantly higher ($p < 0.05$) than the initial resting values, while epinephrine approached the resting value. Peak values of norepinephrine, epinephrine and renin activity showed an approximate 10, 6 and 3 fold increase respectively in the adult group (fig. 2), whereas norepinephrine and epinephrine showed respective increases of 20 and 10 fold in the boys. The plasma renin activity was at a significantly higher level in the boys during all conditions (4 μg/l · h at rest supine before and 15 μg/l · h immediately after work compared with 1.5 and 3.5 μg/l · h respectively in the adults), and showed a steeper increase.



HR	67± 8	84±16	127±19	160±16	179±12	184± 6	126±16	96±11 min^{-1}
SBP	135±13	127±21	164±13	186±16	193±13	188± 8	180±14	131± 7 mmHg
DBP	75±24	83±27	67±34	63±32	65±30	80± 0	63±32	82± 7 mmHg

Fig. 2. Means, standard deviations and ranges of norepinephrine, epinephrine and plasma renin activity at rest in the supine (Sup.) and upright (Upr.) position as well as during and after steady state exercise in 16 young healthy adults. HR = heart rate (means and standard deviations), SBP = systolic blood pressure, DBP = diastolic blood pressure.



HR	96± 6	89±14	132±10	157±15	182±14	198±11	159±15	100± 6 min^{-1}
SBP	130±18	128±19	157±14	167±12	183±13	215± 4	195±16	124±12 mmHg
DBP	72± 5	84± 6	67± 9	66± 8	65± 8	40±29	26±30	75± 6 mmHg

Fig. 3. Means and standard deviations of norepinephrine, epinephrine and plasma renin activity at rest in the supine (Sup.) and upright (Upr.) position as well as during and after steady state exercise in 9 well-trained boys aged 15–16 years. Broken lines indicate ranges of norepinephrine, epinephrine and plasma renin activity as found in the group of healthy young adults (Fig. 2). HR = heart rate (means and standard deviations), SBP = systolic blood pressure, DBP = diastolic blood pressure.

Tab. 1. Plasma dopamine (means, standard deviations and ranges) at rest supine and after standing for 2 min in the upright posture as well as during 4 increasing levels of steady state sitting bicycle ergometer exercise and immediately (0) and 10 min after exercise. For corresponding heart rates and arterial blood pressures see Figs. 2 and 3.

Dopamine (nmol/l)	Rest supine	Rest upright	♀ 35 W ♂ 50 W	♀ 70 W ♂ 100 W	♀ 135 W ♂ 200 W	♀ 170 W ♂ 250 W	0 min	10 min
Adults	0.72 ± 0.59	0.88 ± 0.67	0.86 ± 0.69	0.93 ± 0.76	1.41 ± 0.84	1.49 ± 0.98	1.89 ± 0.97	1.24 ± 0.83
	0.05 - 1.82	0.19 - 2.37	0.22 - 2.26	0.15 - 3.08	0.50 - 2.75	0.72 - 3.18	1.10 - 4.36	0.41 - 3.02
Boys	0.68 ± 0.36	0.93 ± 0.64	0.84 ± 0.42	0.89 ± 0.39	1.11 ± 0.40	1.93 ± 0.48	2.14 ± 0.67	1.45 ± 0.44
	0.23 - 1.20	0.27 - 2.20	0.42 - 1.50	0.23 - 1.50	0.53 - 1.70	1.38 - 2.60	1.28 - 3.20	0.86 - 2.10

Plasma dopamine showed the same general pattern of change under different conditions as epinephrine and norepinephrine; while resting dopamine was in the same range as basal epinephrine, peak values were, however, significantly lower, corresponding to only a 2–3 fold increase of resting dopamine values. There was no significant difference between adults and boys.

Maximal heart rates were significantly higher (198 ± 11) in the boys than in the adults (184 ± 5) while there were only slight differences in systolic and diastolic blood pressures; systolic blood pressures tended to be slightly higher in relation to heart rate in the adults during submaximal work.

Discussion

Catecholamine assay

The major advantages of the assay described are the possibility of determining simultaneously epinephrine, norepinephrine and dopamine, and the small amount of plasma required for a complete analysis of the 3 catecholamines in duplicate. Moreover, there is only minimal (less than 3%) overlap between the different amines on the thin layer plate, indicating a high degree of specificity. The method determines amines in the range of 0.1 nmol/l, and thus achieves a marked improvement in sensitivity compared with the radioenzymatic method initially described by *Engelman* et al, (3) and *Passon & Peuler* (4).

While the specificity and reproducibility are comparable with those achieved by the modification according to *Da Prada & Zürcher* (5) the sensitivity is somewhat lower. However, an important advantage of the method described is its greater simplicity. This was achieved by suppressing all washing and re-extraction procedures aimed at purifying the methylated amines and by omitting the final oxidation of metanephrine and normetanephrine to vanillin. However, this simplification had to be compensated for by lengthening the total period of chromatographic separation to as much as 10 hours and by using a two-dimensional chromatographic procedure. The significant lengthening of the time required to perform the assay is the major disadvantage of the present modification. By using night time for the chromatographic separation the effect of its extension on the total number of samples that can be assayed during a two-day period is, however, minimized.

Obviously, the reproducibility of the method increases significantly with increasing catecholamine levels; hence, determination of plasma catecholamines after a proper adrenergic stimulus appears preferable. Thus the method appears particularly suitable for the study of adrenergic activity in association with physical exercise and, presumably, with intensive emotional stress.

Exercise adrenergic activity in adults and boys

Epinephrine is mainly synthesized in the adrenal medulla. Increased plasma epinephrine levels are thus indicative of increased adreno-medullar secretion. Norepinephrine stems mainly but not exclusively from the postganglionic sympathetic nerve endings; it is stored in the dense core vesicles, together with small amounts of epinephrine (norepinephrine/epinephrine ratio about 5/1). Norepinephrine constitutes the chemical transmitter at most sympathetic postganglionic nerve endings. The release of norepinephrine into the synaptic cleft is proportional to the rate of oncoming sympathetic impulses; at low impulse rates, most (about 90%) of the small amounts released are removed by reuptake into the vesicles: greater amounts of norepinephrine are released into the blood stream only at high impulse rates. These specific properties explain why resting (basal) catecholamine levels are low and show only very small changes (less than 10%) at repeated measurements, provided the conditions are not grossly altered (*Koch*, unpublished observations). It follows that plasma catecholamine levels are more reliable as an indicator of total adrenergic activity the more intensive the adrenergic stimulus.

As could be expected, both plasma catecholamines and plasma renin activity increase concomitantly with increasing work load, the rate of increase being higher the higher the work load. Increased levels of total plasma catecholamines (8) and of norepinephrine (9) have previously been observed in connection with exercise. The absence of detectable levels of epinephrine in the series of *Häggendahl* et al (9) is probably due to the obvious lack of sensitivity and specificity of the fluorometric method used by the authors. In the present study a similar pattern of increase of norepinephrine, epinephrine and plasma renin activity was observed in both groups of subjects, though the rate of norepinephrine increase was much higher than that of epinephrine, particularly at work loads exceeding 60—70% of the maximal work capacity.

It is noteworthy that the boys had higher peak catecholamine levels, in particular of norepinephrine, and higher plasma renin activity levels under all conditions. This probably reflects an age correlated higher peak sympathetic activity in the boys. However, a higher relative work intensity in the boys (maximal heart rate 198 ± 12 as opposed to 184 ± 6 in the adults) is presumably a contributory factor. Results obtained in different types of patients have shown that adrenergic activity in terms of circulating catecholamines is related to the relative work load.

Thus, in patients with severe ischemic heart disease catecholamine levels of the same magnitude as the present peak values have been observed at much lower absolute work loads and heart rates, which, however, corresponded to the near-maximal work load and heart rate of the individual patients (*Koch*, unpublished observations). Young hypertensive patients, on the other hand, did not significantly differ from young normotensives with respect to peak plasma norepinephrine as measured during exercise at near-maximal work loads, but showed significantly higher resting values, and, in particular, a steeper increase of norepinephrine at low and moderate work loads (10).

Recently it has become evident that quite a few pharmacological agents that interfere with hemodynamic regulation, such as α- and β-receptor blockers (11), vasodilators and other antihypertensives, and calcium antagonists (12) also affect and modulate levels of circulating epinephrine and/or norepinephrine during both rest and exercise. While in most cases either the exact mechanism by which these changes occur,

or their physiological significance is not precisely known, it is probable that they have important clinical and therapeutical implications (11). In the few instances where plasma dopamine was also determined it was not found to be significantly changed after acute or short-term treatment with agents such as β-receptors (11), α-β-receptor blockers (13), and calcium antagonists (12). Inversely, long-term treatment with the selective β_1-receptor blocking agent metoprolol has recently been reported to induce an approximate 10–15 fold increase in plasma dopamine (14). The authors suggested that the dopamine increase could be an important factor contributing to the increase in stroke volume and cardiac output generally seen after longterm compared with acute β-receptor blockade. Apparently, the availability of a sensitive method for the determination of plasma catecholamines has not only significantly facilitated and promoted the study of the role and variations of adrenergic stimulation during physiological and pathophysiological conditions, but it has also opened up the possibility of investigating the interaction of pharmacological interventions with the adrenosympathetic system.

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